1   <u>INDIVIDUALIZED ANTI-CANCER ANTIBODIES</u>

2   **Reference to Related Applications:**

3   This application is a continuation of application S.N.

4   09/415,278, filed October 8, 1999, now U.S. Patent

5   _____, the contents of which are herein incorporated

6   by reference.

7   **Field of the Invention:**

8   This invention relates to the production of anti-cancer

9   antibodies customized for the individual patient that can be

10   used for therapeutic and diagnostic purposes. The invention

11   further relates to the process by which the antibodies are

12   made and to their methods of use.

13

14   **Background of the Invention:**

15   Each individual who presents with cancer is unique and

16   has a cancer that is as different from other cancers as that

17   person's identity. Despite this, current therapy treats all

18   patients with the same type of cancer, at the same stage, in

19   the same way. At least 30% of these patients will fail the

20   first line therapy, thus leading to further rounds of

21   treatment and the increased probability of treatment failure,

22   metastases, and ultimately, death. A superior approach to

23   treatment would be the customization of therapy for the

24   particular individual. The only current therapy which lends

1   itself to customization is surgery. Chemotherapy and

2   radiation treatment can not be tailored to the patient, and

3   surgery by itself, in most cases is inadequate for producing

4   cures.

5   With the advent of monoclonal antibodies, the

6   possibility of developing methods for customized therapy

7   became more realistic since each antibody can be directed to

8   a single epitope. Furthermore, it is possible to produce a

9   combination of antibodies that are directed to the

10   constellation of epitopes that uniquely define a particular

11   individual's tumor.

12   Having recognized that a significant difference between

13   cancerous and normal cells is that cancerous cells contain

14   antigens that are specific to transformed cells, the

15   scientific community has long held that monoclonal antibodies

16   can be designed to specifically target transformed cells by

17   binding specifically to these cancer antigens; thus giving

18   rise to the belief that monoclonal antibodies can serve as

19   "Magic Bullets" to eliminate cancer cells.

20   At the present time, however, the cancer patient usually

21   has few options of treatment. The regimented approach to

22   cancer therapy has produced improvements in global survival

23   and morbidity rates. However, to the particular individual,

24   these improved statistics do not necessarily correlate with

25   an improvement in their personal situation.

1   Thus, if a methodology was put forth which enabled the

2   practitioner to treat each tumor independently of other

3   patients in the same cohort, this would permit the unique

4   approach of tailoring therapy to just that one person. Such

5   a course of therapy would, ideally, increase the rate of

6   cures, and produce better outcomes, thereby satisfying a

7   long-felt need.

8   Historically, the use of polyclonal antibodies has been

9   used with limited success in the treatment of human cancers.

10   Lymphomas and leukemias have been treated with human plasma,

11   but there were few prolonged remission or responses.

12   Furthermore, there was a lack of reproducibility and there

13   was no additional benefit compared to chemotherapy. Solid

14   tumors such as breast cancers, melanomas and renal cell

15   carcinomas have also been treated with human blood,

16   chimpanzee serum, human plasma and horse serum with

17   correspondingly unpredictable and ineffective results.

18   There have been many clinical trials of monoclonal

19   antibodies for solid tumors. In the 1980s there were at least

20   four clinical trials for human breast cancer which produced

21   only one responder from at least 47 patients using antibodies

22   against specific antigens or based on tissue selectivity. It

23   was not until 1998 that there was a successful clinical trial

24   using a humanized anti-her 2 antibody in combination with

25   Cisplatin. In this trial 37 patients were accessed for

1 responses of which about a quarter had a partial response

2 rate and another half had minor or stable disease

3 progression.

4 The clinical trials investigating colorectal cancer

5 involve antibodies against both glycoprotein and glycolipid

6 targets. Antibodies such as 17-1A, which has some

7 specificity for adenocarcinomas, had undergone Phase 2

8 clinical trials in over 60 patients with only one patient

9 having a partial response. In other trials, use of 17-1A

10 produced only one complete response and two minor responses

11 among 52 patients in protocols using additional

12 cyclophosphamide. Other trials involving 17-1A yielded

13 results that were similar. The use of a humanized murine

14 monoclonal antibody initially approved for imaging also did

15 not produce tumor regression. To date there has not been an

16 antibody that has been effective for colorectal cancer.

17 Likewise there have been equally poor results for lung

18 cancer, brain cancers, ovarian cancers, pancreatic cancer,

19 prostate cancer, and stomach cancer. There has been some

20 limited success in the use of anti-GD3 monoclonal antibody

21 for melanoma. Thus, it can be seen that despite successful

22 small animal studies that are a prerequisite for human

23 clinical trials, the antibodies that have been tested have

24 been for the most part ineffective.

25 **Prior Patents:**

1   U.S. Patent No. 5,750,102 discloses a process wherein

2 cells from a patient's tumor are transfected with MHC genes

3 which may be cloned from cells or tissue from the patient.

4 These transfected cells are then used to vaccinate the

5 patient.

6   U.S. Patent No. 4,861,581 discloses a process comprising

7 the steps of obtaining monoclonal antibodies that are

8 specific to an internal cellular component of neoplastic and

9 normal cells of the mammal but not to external components,

10 labeling the monoclonal antibody, contacting the labeled

11 antibody with tissue of a mammal that has received therapy to

12 kill neoplastic cells, and determining the effectiveness of

13 therapy by measuring the binding of the labeled antibody to

14 the internal cellular component of the degenerating

15 neoplastic cells. In preparing antibodies directed to human

16 intracellular antigens, the patentee recognizes that

17 malignant cells represent a convenient source of such

18 antigens.

19   U.S. Patent No. 5,171,665 provides a novel antibody and

20 method for its production. Specifically, the patent teaches

21 formation of a monoclonal antibody which has the property of

22 binding strongly to a protein antigen associated with human

23 tumors, e.g. those of the colon and lung, while binding to

24 normal cells to a much lesser degree.

1   U.S. Patent No. 5,484,596 provides a method of cancer

2   therapy comprising surgically removing tumor tissue from a

3   human cancer patient, treating the tumor tissue to obtain

4   tumor cells, irradiating the tumor cells to be viable but

5   non-tumorigenic, and using these cells to prepare a vaccine

6   for the patient capable of inhibiting recurrence of the

7   primary tumor while simultaneously inhibiting metastases.

8   The patent teaches the development of monoclonal antibodies

9   which are reactive with surface antigens of tumor cells. As

10 set forth at col. 4, lines 45 et seq., the patentees utilize

11 autochthonous tumor cells in the development of monoclonal

12 antibodies expressing active specific immunotherapy in human

13 neoplasia.

14   U.S. Patent No. 5,693,763 teaches a glycoprotein antigen

15 characteristic of human carcinomas and not dependent upon the

16 epithelial tissue of origin.

17   U.S. Patent No. 5,783,186 is drawn to Anti-Her2

18 antibodies which induce apoptosis in Her2 expressing cells,

19 hybridoma cell lines producing the antibodies, methods of

20 treating cancer using the antibodies and pharmaceutical

21 compositions including said antibodies.

22   U.S. Patent No. 5,849,876 describes new hybridoma cell

23 lines for the production of monoclonal antibodies to mucin

24 antigens purified from tumor and non-tumor tissue sources.

1   U.S. Patent No. 5,869,268 is drawn to a method for

2   producing a human lymphocyte producing an antibody specific

3   to a desired antigen, a method for producing a monoclonal

4   antibody, as well as monoclonal antibodies produced by the

5   method. The patent is particularly drawn to the production

6   of an anti-HD human monoclonal antibody useful for the

7   diagnosis and treatment of cancers.

8   U.S. Patent No. 5,869,045 relates to antibodies,

9   antibody fragments, antibody conjugates and single chain

10   immunotoxins reactive with human carcinoma cells. The

11   mechanism by which these antibodies function is two-fold, in

12   that the molecules are reactive with cell membrane antigens

13   present on the surface of human carcinomas, and further in

14   that the antibodies have the ability to internalize within

15   the carcinoma cells, subsequent to binding, making them

16   especially useful for forming antibody-drug and antibody-

17   toxin conjugates. In their unmodified form the antibodies

18   also manifest cytotoxic properties at specific

19   concentrations.

20   U.S. Patent No. 5,780,033 discloses the use of

21   autoantibodies for tumor therapy and prophylaxis. However,

22   this antibody is an antinuclear autoantibody from an aged

23   mammal. In this case, the autoantibody is said to be one type

24   of natural antibody found in the immune system. Because the

25   autoantibody comes from "an aged mammal", there is no

1 requirement that the autoantibody actually comes from the

2 patient being treated. In addition the patent discloses

3 natural and monoclonal antinuclear autoantibody from an aged

4 mammal, and a hybridoma cell line producing a monoclonal

5 antinuclear autoantibody.

6

Summary of the Invention:

8 This application teaches a method for producing patient

9 specific anti-cancer antibodies using a novel paradigm of

10 screening. These antibodies can be made specifically for one

11 tumor and thus make possible the customization of cancer

12 therapy. Within the context of this application, anti-cancer

13 antibodies having either cell-killing (cytotoxic) or cell-

14 growth inhibiting (cytostatic) properties will hereafter be

15 referred to as cytotoxic. These antibodies can be used in

16 aid of staging and diagnosis of a cancer, and can be used to

17 treat tumor metastases.

18 The prospect of individualized anti-cancer treatment

19 will bring about a change in the way a patient is managed. A

20 likely clinical scenario is that a tumor sample is obtained

21 at the time of presentation, and banked. From this sample,

22 the tumor can be typed from a panel of pre-existing anti-

23 cancer antibodies. The patient will be conventionally staged

24 but the available antibodies can be of use in further staging

25 the patient. The patient can be treated immediately with the

1 existing antibodies, and a panel of antibodies specific to

2 the tumor can be produced either using the methods outlined

3 herein or through the use of phage display libraries in

4 conjunction with the screening methods herein disclosed. All

5 the antibodies generated will be added to the library of

6 anti-cancer antibodies since there is a possibility that

7 other tumors can bear some of the same epitopes as the one

8 that is being treated.

9 In addition to anti-cancer antibodies, the patient can

10 elect to receive the currently recommended therapies as part

11 of a multi-modal regimen of treatment. The fact that the

12 antibodies isolated via the present methodology are

13 relatively non-toxic to non-cancerous cells allow

14 combinations of antibodies at high doses to be used, either

15 alone, or in conjunction with conventional therapy. The high

16 therapeutic index will also permit re-treatment on a short

17 time scale that should decrease the likelihood of emergence

18 of treatment resistant cells.

19 If the patient is refractory to the initial course of

20 therapy or metastases develop, the process of generating

21 specific antibodies to the tumor can be repeated for re-

22 treatment. Furthermore, the anti-cancer antibodies can be

23 conjugated to red blood cells obtained from that patient and

24 re-infused for treatment of metastases. There have been few

25 effective treatments for metastatic cancer and metastases

1   usually portend a poor outcome resulting in death. However,

2   metastatic cancers are usually well vascularized and the

3   delivery of anti-cancer antibodies by red blood cells can

4   have the effect of concentrating the antibodies at the site

5   of the tumor. Even prior to metastases, most cancer cells

6   are dependent on the host's blood supply for their survival

7   and anti-cancer antibody conjugated red blood cells can be

8   effective against *in situ* tumors, too. Alternatively, the

9   antibodies may be conjugated to other hematogenous cells,

10   e.g. lymphocytes, macrophages, monocytes, natural killer

11   cells, etc.

12   There are five classes of antibodies and each is

13   associated with a function that is conferred by its heavy

14   chain. It is generally thought that cancer cell killing by

15   naked antibodies are mediated either through antibody

16   dependent cellular cytotoxicity or complement dependent

17   cytotoxicity. For example murine IgM and IgG2a antibodies

18   can activate human complement by binding the C-1 component of

19   the complement system thereby activating the classical

20   pathway of complement activation which can lead to tumor

21   lysis. For human antibodies the most effective complement

22   activating antibodies are generally IgM and IgG1. Murine

23   antibodies of the IgG2a and IgG3 isotype are effective at

24   recruiting cytotoxic cells that have Fc receptors which will

25   lead to cell killing by monocytes, macrophages, granulocytes

1 and certain lymphocytes. Human antibodies of both the IgG1

2 and IgG3 isotype mediate ADCC.

3 Another possible mechanism of antibody mediated cancer

4 killing may be through the use of antibodies that function to

5 catalyze the hydrolysis of various chemical bonds in the cell

6 membrane and its associated glycoproteins or glycolipids, so-

7 called catalytic antibodies.

8 There are two additional mechanisms of antibody mediated

9 cancer cell killing which are more widely accepted. The

10 first is the use of antibodies as a vaccine to induce the

11 body to produce an immune response against the putative

12 cancer antigen that resides on the tumor cell. The second is

13 the use of antibodies to target growth receptors and

14 interfere with their function or to down regulate that

15 receptor so that effectively its function is lost.

16 Accordingly, it is an objective of the invention to

17 teach a method for producing anti-cancer antibodies from

18 cells derived from a particular individual which are

19 cytotoxic with respect to cancer cells while simultaneously

20 being relatively non-toxic to non-cancerous cells.

21 It is an additional objective of the invention to

22 produce novel anti-cancer antibodies.

23 It is a further objective of the instant invention to

24 produce anti-cancer antibodies whose cytotoxicity is mediated

25 through antibody dependent cellular toxicity.

1   It is yet an additional objective of the instant

2   invention to produce anti-cancer antibodies whose

3   cytotoxicity is mediated through complement dependent

4   cellular toxicity.

5   It is still a further objective of the instant invention

6   to produce anti-cancer antibodies whose cytotoxicity is a

7   function of their ability to catalyze hydrolysis of cellular

8   chemical bonds.

9   Still an additional objective of the instant invention

10   is to produce anti-cancer antibodies useful as a vaccine to

11   produce an immune response against putative cancer antigen

12   residing on tumor cells.

13   A further objective of the instant invention is the use

14   of antibodies to target cell membrane proteins, such as

15   growth receptors, cell membrane pumps and cell anchoring

16   proteins, thereby interfering with or down regulating their

17   function.

18   Yet an additional objective of the instant invention is

19   the production of anti-cancer antibodies whose cell-killing

20   utility is concomitant with their ability to effect a

21   conformational change in cellular proteins such that a signal

22   will be transduced to initiate cell-killing.

23   A still further objective of the instant invention is to

24   produce anti-cancer antibodies which are useful for

25   diagnosis, prognosis, and monitoring of cancer, e.g.

1   production of a panel of therapeutic anti-cancer antibodies

2   to test patient samples to determine if they contain any

3   suitable antibodies for therapeutic use.

4   Yet another objective of the instant invention is to

5   produce novel antigens, associated with cancer processes,

6   which can be discovered by using anti-cancer antibodies

7   derived by the process of the instant invention. These

8   antigens are not limited to proteins, as is generally the

9   case with genomic data; they may also be derived from

10   carbohydrates or lipids or combinations thereof.

11   Other objects and advantages of this invention will

12   become apparent from the following description wherein are

13   set forth, by way of illustration and example, certain

14   embodiments of this invention.

15

16   **Detailed Description of the Invention:**

17   It is to be understood that while a certain form of the

18   invention is illustrated, it is not to be limited to the

19   specific form or arrangement herein described and shown. It

20   will be apparent to those skilled in the art that various

21   changes may be made without departing from the scope of the

22   invention and the invention is not to be considered limited

23   to what is shown and described in the specification.

24   One of the potential benefits of monoclonal antibodies

25   with respect to the treatment of cancer is their ability to

1 specifically recognize single antigens. It was thought that

2 in some instances cancer cells possess antigens that were

3 specific to that kind of transformed cell. It is now more

4 frequently believed that cancer cells have few unique

5 antigens, rather, they tend to over-express a normal antigen

6 or express fetal antigens. Nevertheless, the use of

7 monoclonal antibodies provided a method of delivering

8 reproducible doses of antibodies to the patient with the

9 expectation of better response rates than with polyclonal

10 antibodies.

11 Traditionally, monoclonal antibodies have been made

12 according to fundamental principles laid down by Kohler and

13 Milstein. Mice are immunized with antigens, with or without,

14 adjuvants. The splenocytes are harvested from the spleen for

15 fusion with immortalized hybridoma partners. These are

16 seeded into microtitre plates where they can secrete

17 antibodies into the supernatant that is used for cell

18 culture. To select from the hybridomas that have been plated

19 for the ones that produce antibodies of interest the

20 hybridoma supernatants are usually tested for antibody

21 binding to antigens in an ELISA (enzyme linked immunosorbent

22 assay) assay. The idea is that the wells that contain the

23 hybridoma of interest will contain antibodies that will bind

24 most avidly to the test antigen, usually the immunizing

25 antigen. These wells are then subcloned in limiting dilution

26 fashion to produce monoclonal hybridomas. The selection for

1   the clones of interest is repeated using an ELISA assay to

2   test for antibody binding. Therefore, the principle that has

3   been propagated is that in the production of monoclonal

4   antibodies the hybridomas that produce the most avidly

5   binding antibodies are the ones that are selected from among

6   all the hybridomas that were initially produced. That is to

7   say, the preferred antibody is the one with highest affinity

8   for the antigen of interest.

9   There have been many modifications of this procedure

10 such as using whole cells for immunization. In this method,

11 instead of using purified antigens, entire cells are used for

12 immunization. Another modification is the use of cellular

13 ELISA for screening. In this method instead of using

14 purified antigens as the target in the ELISA, fixed cells are

15 used. In addition to ELISA tests, complement mediated

16 cytotoxicity assays have also been used in the screening

17 process. However, antibody-binding assays were used in

18 conjunction with cytotoxicity tests. Thus, despite many

19 modifications, the process of producing monoclonal antibodies

20 relies on antibody binding to the test antigen as an

21 endpoint.

22   Most antibodies directed against cancer cells have been

23 produced using the traditional methods outlined above. These

24 antibodies have been used both therapeutically and

25 diagnostically. In general, for both these applications, the

1 antibody has been used as the targeting agent that delivers a

2 payload to the site of the cancer. These antibody conjugates

3 can either be radioactive, toxic, or serve as an intermediary

4 for further delivery of a drug to the body, such as an enzyme

5 or biotin. Furthermore, it was widely held, until recently,

6 that naked antibodies had little effect *in vivo*. Both

7 HERCEPTIN and RITUXIMAB are humanized murine monoclonal

8 antibodies that have recently been approved for human use by

9 the FDA. However, both these antibodies were initially made

10 by assaying for antibody binding and their direct

11 cytotoxicity was not the primary goal during the production

12 of hybridomas. Any tendency for these antibodies to produce

13 tumor cell killing is thus through chance, not by design.

14 Although the production of monoclonal antibodies have

15 been carried out using whole cell immunization for various

16 applications the screening of these hybridomas have relied on

17 either putative or identified target antigens or on the

18 selectivity of these hybridomas for specific tissues. It is

19 axiomatic that the best antibodies are the ones with the

20 highest binding constants. This concept originated from the

21 basic biochemical principle that enzymes with the highest

22 binding constants were the ones that were the most effective

23 for catalyzing a reaction. This concept is applicable to

24 receptor ligand binding where the drug molecule binding to

25 the receptor with the greatest affinity usually has the

26 highest probability for initiating or inhibiting a signal.

1   However, this may not always be the case since it is possible

2   that in certain situations there may be cases where the

3   initiation or inhibition of a signal may be mediated through

4   non-receptor binding. The information conveyed by a

5   conformational change induced by ligand binding can have many

6   consequences such as a signal transduction, endocytosis,

7   among the others. The ability to produce a conformational

8   change in a receptor molecule may not necessarily be due to

9   the filling of a ligand receptor pocket but may occur through

10   the binding of another extra cellular domain or due to

11   receptor clustering induced by a multivalent ligand.

12   The production of antibodies to produce cell killing

13   need not be predicated upon screening of the hybridomas for

14   the best binding antibodies. Rather, although not advocated

15   by those who produce monoclonal antibodies, the screening of

16   the hybridoma supernatants for cell killing or alternatively

17   for cessation of growth of the cancerous cells may be

18   selected as a desirable endpoint for the production of

19   cytotoxic or cytostatic antibodies. It is well understood

20   that the *in-vivo* antibodies mediate their function through

21   the Fc portions and that the utility of the therapeutic

22   antibody is determined by the functionality of the constant

23   region or attached moieties. In this case the FAb portion of

24   the antibody, the antigen-combining portion, will confer to

25   the antibody its specificity and the Fc portion its

1   functionality. The antigen combining site of the antibody

2   can be considered to be the product of a natural

3   combinatorial library. The result of the rearrangement of the

4   variable region of the antibody can be considered a molecular

5   combinatorial library where the output is a peptide.

6   Therefore, the sampling of this combinatorial library can be

7   based on any parameter. Like sampling a natural compound

8   library for antibiotics, it is possible to sample an antibody

9   library for cytotoxic or cytostatic compounds.

10   The various endpoints in a screen must be differentiated

11   from each other. For example, the difference between antibody

12   binding to the cell is distinct from cell killing. Cell

13   killing (cytotoxicity) is distinct from the mechanisms of

14   cell death such as oncosis or apoptosis. There can be many

15   processes by which cell death is achieved and some of these

16   can lead either to oncosis or apoptosis. There is speculation

17   that there are other cell death mechanisms other than oncosis

18   or apoptosis but regardless of how the cell arrives at death

19   there are some commonalities of cell death. One of these is

20   the absence of metabolism and another is the denaturation of

21   enzymes. In either case vital stains will fail to stain these

22   cells. These endpoints of cell death have been long

23   understood and predate the current understanding of the

24   mechanisms of cell death. Furthermore, there is the

25   distinction between cytotoxic effects where cells are killed

1   and cytostatic effects where the proliferation of cells are

2   inhibited.

3   In a preferred embodiment of the present invention, the

4   assay is conducted by focusing on cytotoxic activity toward

5   cancerous cells as an end point. In a preferred embodiment,

6   a live /dead assay kit , for example the LIVE/DEAD®

7   Viability/Cytotoxicity Assay Kit (L-3224) by Molecular

8   Probes, is utilized. The Molecular Probes kit provides a

9   two-color fluorescence cell viability assay that is based on

10 the simultaneous determination of live and dead cells with

11 two probes that measure two recognized parameters of cell

12 viability — intracellular esterase activity and plasma

13 membrane integrity. The assay principles are general and

14 applicable to most eukaryotic cell types, including adherent

15 cells and certain tissues, but not to bacteria or yeast.

16 This fluorescence-based method of assessing cell viability is

17 preferred in place of such assays as trypan blue exclusion,

18 Cr release and similar methods for determining cell viability

19 and cytotoxicity.

20   In carrying out the assay, live cells are distinguished

21 by the presence of ubiquitous intracellular esterase

22 activity, determined by the enzymatic conversion of the

23 virtually nonfluorescent cell-permeant CALCEIN AM to the

24 intensely fluorescent Calcein. The polyanionic dye Calcein

25 is well retained within live cells, producing an intense

uniform green fluorescence in live cells (ex/em ~495 nm/~515 nm). EthD-1 enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells (ex/em ~495 nm/~635 nm). EthD-1 is excluded by the intact plasma membrane of live cells. The determination of cell viability depends on these physical and biochemical properties of cells. Cytotoxic events that do not affect these cell properties may not be accurately assessed using this method. Background fluorescence levels are inherently low with this assay technique because the dyes are virtually nonfluorescent before interacting with cells.

In addition to the various endpoints for screening, there are two other major characteristics of the screening process. The library of antibody gene products is not a random library but is the product of a biasing procedure. In the examples below, the biasing is produced by immunizing mice with fixed cells. This increases the proportion of antibodies that have the potential to bind the target antigen. Although immunization is thought of as a way to produce higher affinity antibodies (affinity maturation) in this case it is not. Rather, it can be considered as a way to shift the set of antigen combining sites towards the targets. This is also distinct from the concept of isotype switching where the functionality, as dictated by the

1   constant portion of the heavy chain, is altered from the

2   initial IgM isotype to another isotype such as IgG.

3   The third key feature that is crucial in the screening

4   process is the use of multitarget screening. To a certain

5   extent specificity is related to affinity. An example of this

6   is the situation where an antigen has very limited tissue

7   distribution and the affinity of the antibody is a key

8   determinant of the specificity of the antibody-the higher the

9   affinity the more tissue specific the antibody and likewise

10 an antibody with low affinity may bind to tissues other than

11 the one of interest. Therefore, to address the specificity

12 issue the antibodies are screened simultaneously against a

13 variety of cells. In the examples below the hybridoma

14 supernatants (representing the earliest stages of monoclonal

15 antibody development), are tested against a number of cell

16 lines to establish specificity as well as activity.

17   The antibodies are designed for therapeutic treatment of

18 cancer in patients. Ideally the antibodies can be naked

19 antibodies. They can also be conjugated to toxins. They can

20 be used to target other molecules to the cancer. e.g. biotin

21 conjugated enzymes. Radioactive compounds can also be used

22 for conjugation.

1   The antibodies can be fragmented and rearranged

2   molecularly. For example Fv fragments can be made; sFv-single

3   chain Fv fragments; diabodies etc.

4   It is envisioned that these antibodies can be used for

5   diagnosis, prognosis, and monitoring of cancer. For example

6   the patients can have blood samples drawn for shed tumor

7   antigens which can be detected by these antibodies in

8   different formats such as ELISA assays, rapid test panel

9   formats etc. The antibodies can be used to stain tumor

10   biopsies for the purposes of diagnosis. In addition a panel

11   of therapeutic antibodies can be used to test patient samples

12   to determine if there are any suitable antibodies for

13   therapeutic use.

14   **Example one**

15   In order to produce monoclonal antibodies specific for a

16   tumor sample the method of selection of the appropriate

17   hybridoma wells is complicated by the probability of

18   selecting wells which will produce false positive signals.

19   That is to say that there is the likelihood of producing

20   antibodies that can react against normal cells as well as

21   cancer cells. To obviate this possibility one strategy is to

22   mask the anti-normal antigen antibodies from the selection

23   process. This can be accomplished by removing the anti-

24   normal antibodies at the first stage of screening thereby

1 revealing the presence of the desired antibodies. Subsequent

2 limiting dilution cloning can delineate the clones that will

3 not produce killing of control cells but will produce target

4 cancer cell killing.

5 Biopsy specimens of breast, melanoma, and lung tumors

6 were obtained and stored at -70°C until used. Single cell

7 suspensions were prepared and fixed with -30°C, 70% ethanol,

8 washed with PBS and reconstituted to an appropriate volume

9 for injection. Balb/c mice were immunized with $2.5 \times 10^5 - 1 \times 10^6$

10 cells and boosted every third week until a final pre-fusion

11 boost was performed three days prior to the splenectomy. The

12 hybridomas were prepared by fusing the isolated splenocytes

13 with Sp2/0 and NS1 myeloma partners. The supernatants from

14 the fusions were tested for subcloning of the hybridomas.

15 Cells (including A2058 melanoma cells, CCD-12CoN fibroblasts,

16 MCF-12A breast cells among others) were obtained from ATCC

17 and cultured according to enclosed instructions. The HEY cell

18 line was a gift from Dr. Inka Brockhausen. The non-cancer

19 cells, e.g. CCD-12CoN fibroblasts and MCF-12A breast cells,

20 were plated into 96-well microtitre plates (NUNC) 1 to 2

21 weeks prior to screening. The cancer cells, e.g. HEY, A2058,

22 BT 483, and HS294t, were plated two or three days prior to

23 screening.

24 The plated normal cells were fixed prior to use. The

25 plates were washed with 100 microliters of PBS for 10 minutes

1 at room temperature and then aspirated dry. 75 microliters

2 of 0.01 percent glutaraldehyde diluted in PBS were added to

3 each well for five minutes and then aspirated. The plates

4 were washed with 100 microliters of PBS three times at room

5 temperature. The wells were emptied and 100 microliters of

6 one percent human serum albumin in PBS was added to each well

7 for one hour at room temperature. The plates were then

8 stored at four degrees Celsius.

9 Prior to the transfer of the supernatant from the

10 hybridoma plates the fixed normal cells were washed three

11 times with 100 microliters of PBS at room temperature. After

12 aspiration to the microliters of the primary hybridoma

13 culture supernatants were transferred to the fixed cell

14 plates and incubated for two hours at 37 degrees Celsius in a

15 8 percent CO_2 incubator. The hybridoma supernatants derived

16 from melanoma was incubated with CCD-12 CoN cells and those

17 derived from breast cancer were incubated with MCF-12a cells.

18 After incubation the absorbed supernatant was divided

19 into two 75 microliter portions and transferred to target

20 cancer cell plates. Prior to the transfer the cancer cell

21 plates were washed three times with 100 microliters of PBS.

22 The supernatant from the CCD-12 CoN cells were transferred to

23 the A2058 and the HS294t cells, whereas the supernatant from

24 MCF-12A cells were transferred to the HEY and BT 483 cells.

25 The cancer cells were incubated with the hybridoma

1   supernatants for 18 hours at 37 degrees Celsisu in an 8

2   percent CO_2 incubator.

3   The Live/Dead cytotoxicity assay was obtained from

4   Molecular Probes (Eu,OR). The assays were performed according

5   to the manufacturer's instructions with the changes outlined

6   below. The plates with the cells were washed once with 100

7   microliters of PBS at 37°C. 75 to 100 microliters of

8   supernatant from the hybridoma microtitre plates were

9   transferred to the cell plates and incubated in a 8% CO_2

10   incubator for 18-24 hours. Then, the wells that served as the

11   all dead control were aspirated until empty and 50

12   microliters of 70% ethanol was added. The plate was then

13   emptied by inverting and blotted dry. Room temperature PBS

14   was dispensed into each well from a multichannel squeeze

15   bottle, tapped three times, emptied by inversion and then

16   blotted dry. 50 microliters of the fluorescent Live/Dead dye

17   diluted in PBS was added to each well and incubated at 37°C

18   in a 5% CO_2 incubator for one hour. The plates were read in a

19   Perkin-Elmer HTS7000 fluorescence plate reader and the data

20   was analyzed in Microsoft Excel.

21   Four rounds of screening were conducted to produce

22   single clone hybridoma cultures. For two rounds of screening

23   the hybridoma supernatants were tested only against the

24   cancer cells. In the last round of screening the supernatant

25   was tested against a number of non-cancer cells as well as

1   the target cells indicated in table 1. The antibodies were

2   isotyped using a commercial isotyping kit.

3   A number of monoclonal antibodies were produced in

4   accordance with the method of the present invention. These

5   antibodies, whose characteristics are summarized in Table 1,

6   are identified as 3BD-3, 3BD-6, 3BD-8, 3BD-9, 3BD-15, 3BD-25,

7   3BD-26 and 3BD-27. Each of the designated antibodies is

8   produced by a hybridoma cell line deposited with the American

9   Type Culture Collection at 10801 University Boulevard,

10   Manassas, Va. having an ATCC Accession Number as follows:

11   **Antibody**   **ATCC Accession Number**

12   3BD-3

13   3BD-6

14   3BD-8

15   3BD-9

16   3BD-15

17   3BD-25

18   3BD-26

19   3BD-27

20   These antibodies are considered monoclonal after four rounds

21   of limiting dilution cloning. The anti-melanoma antibodies

22   did not produce significant cancer cell killing. The panel of

23   anti-breast cancer antibodies killed 32-87% of the target

24   cells and <1-3% of the control cells. The predominant isotype

1   was IgG1 even though it was expected that the majority of

2   anti-tumor antibodies would be directed against carbohydrate

3   antigens, and thus, be of the IgM type. There is a high

4   therapeutic index since most antibodies spare the control

5   cells from cell death.

6   Table 1. Anti-Breast Cancer Antibodies

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Clones	% Cell Death			
	Target for Anti-Breast Cancer Antibodies (HEY & A2058)	Normal Fibroblast Cells (CCD-12CoN)	Fibrocystic Breast Cells (MCF-12A)	Isotype
3BD-3	74.9%	3.7%	<1%	γ1, λ
3BD-6	68.5%	5.6%	<1%	γ1, λ
3BD-8	81.9%	4.5%	2.6%	γ1, κ
3BD-9	77.2%	7.9%	<1%	γ1, λ
3BD-15	87.1%	<1 %	<1%	γ1, λ
3BD-26	54.8%	3.3%	<1%	μ,κ
3BD-25	32.4%	3.6%	<1 %	γ1,κ
3BD-27	60.1%	8.3%	1.3%	γ1, κ

19

20 **Example 2**

21   In this example customized anti-cancer antibodies are

22   produced by first obtaining samples of the patient's tumor.

23   Usually this is from a biopsy specimen from a solid tumor or

24   a blood sample from hematogenous tumors. The samples are

25   prepared into single cell suspensions and fixed for injection

1   into mice. After the completion of the immunization schedule

2   the hybridomas are produced from the splenocytes. The

3   hybridomas are screened against a variety of cancer cell

4   lines and normal cells in standard cytotoxicity assays. Those

5   hybridomas that are reactive against cancer cell lines but

6   are not reactive against normal non-transformed cells are

7   selected for further propagation. Clones that were considered

8   positive were ones that selectively killed the cancer cells

9   but did not kill the non-transformed cells. The antibodies

10 are characterized for a large number of biochemical

11 parameters and then humanized for therapeutic use.

12 The melanoma tumor cells isolated and cell lines were

13 cultured as described in Example 1. Balb/c mice were

14 immunized according to the following schedule: 200,000 cells

15 s.c. and i.p. on day 0, then 200,000 cells were injected i.p.

16 on day 21, then 1,000,000 cells were injected on day 49, then

17 1,250,000 cells in Freund's Complete Adjuvant were injected

18 i.p. on day 107, and then 200,000 cells were injected on day

19 120 i.p. and then the mice were sacrificed on day 123. The

20 spleens were harvested and the splenocytes were divided into

21 two aliquots for fusion with Sp2/0 (1LN) or NS-1 (2LN)

22 myeloma partners using the methods outlined in example 1.

23   The screening was carried out 11 days after the fusion

24 against A2058 melanoma cells and CCD-12CoN fibroblasts. Each

25 pair of plates were washed with 100 microliters of room

1   temperature PBS and then aspirated to near dryness. Then 50

2   microliters of hybridoma supernatant was added to the same

3   wells on each of the two plates. The spent Sp2/0 supernatant

4   was added to the control wells at the same volume and the

5   plates were incubated for around 18 hours at 37 degrees

6   Celsius at a 8%CO_2, 98% relative humidity incubator. Then

7   each pair of plates were removed and in the positive control

8   wells 50 microliters of 70% ethanol was substituted for the

9   media for 4 seconds. The plates were then inverted and washed

10 with room temperature PBS once and dried. Then 50uL of

11 fluorescent live/dead dye diluted in PBS (Molecular Probes

12 Live/Dead Kit) was added for one hour and incubated at 37

13 degrees Celsius. The plates were then read in a Perkin-Elmer

14 fluorescent plate reader and the data analyzed using

15 Microsoft Excel. The wells that were considered positive were

16 subcloned and the same screening process was repeated 13 days

17 later and then 33 days later. The results of the last

18 screening is outlined in Table 2 below. A number of

19 monoclonal antibodies were produced in accordance with the

20 method of the present invention. These antibodies, whose

21 characteristics are summarized in Table 2, are identified as

22 1LN-1, 1LN-12, 1LN-14, 2LN-21, 2LN-28, 2LN-29, 2LN-31, 2LN-

23 33, 2LN-34 and 2LN-35. Each of the designated antibodies is

24 produced by a hybridoma cell line deposited with the American

25 Type Culture Collection at 10801 University Boulevard,

26 Manassas, Va. having an ATCC Accession Number as follows:

	Antibody	ATCC Accession Number
1		
2		
3	**Antibody**	**ATCC Accession Number**
4	1LN-1	
5	1LN-12	
6	1LN-14	
7	2LN-21	
8	2LN-28	
9	2LN-29	
10	2LN-31	
11	2LN-33	
12	2LN-34	
13	2LN-35	

14 Table 2, Anti-Melanoma Antibodies

		% Cell Death	
	Clones	Target for Anti-Melanoma Antibodies (A2058)	Normal Fibroblast Cells (CCD-1 2CoN)
17	1LN-1	59.4%	<1 %
18	1LN-12	55.2%	1 4%
19	1LN-14	51.4%	<1%
20	2LN-21	72.0%	15.9%
21	2LN-28	66.6%	12.4%
22	2LN-29	78.2%	6.1%
23	2LN-31	100%	7.8%
24	2LN-33	94.2%	<1%

2LN-34	56.6%	11.2%
2LN-35	66.5%	6.6%

The table illustrates that clones from both the Sp2/0 and NS-1 fusions were able to produce antibodies that had a greater than 50% killing rate for cancerous cells and at the same time some of the clones were able to produce less than one percent killing of normal control fibroblasts.

The anti-cancer antibodies of the invention are useful for treating a patient with a cancerous disease when administered in admixture with a pharmaceutically acceptable adjuvant, for example normal saline, a lipid emulsion, albumen, phosphate buffered saline or the like and are administered in an amount effective to mediate treatment of said cancerous disease, for example with a range of about 1 microgram per mil to about 1 gram per mil.

The method for treating a patient suffering from a cancerous disease may further include the use of conjugated anti-cancer antibodies and would this include conjugating patient specific anti-cancer antibodies with a member selected from the group consisting of toxins, enzymes, radioactive compounds, and hematogenous cells; and administering these conjugated antibodies to the patient; wherein said anti-cancer antibodies are administered in admixture with a pharmaceutically acceptable adjuvant, for example normal saline, a lipid emulsion, albumen, phosphate

1 buffered saline or the like and are administered in an amount

2 effective to mediate treatment of said cancerous disease, for

3 example with a range of about 1 microgram per mil to about 1

4 gram per mil. In a particular embodiment, the anti-cancer

5 antibodies useful in either of the above outlined methods may

6 be a humanized antibody.

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